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Predicting *Salmonella enterica* serotypes by repetitive sequence-based PCR ☆

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ABSTRACT

Repetitive extragenic palindromic sequence-based PCR (rep-PCR) utilizing a semi-automated system, was evaluated as a method to determine *Salmonella* serotypes. A group of 216 *Salmonella* isolates belonging to 13 frequently isolated serotypes and one rarer serotype from poultry were used to create a DNA fingerprint library with the DiversiLab™ System software. Subsequently, a blinded set of 44 poultry isolates were fingerprinted and queried against the library in an attempt to putatively assign a serotype designation to each *Salmonella* isolate. The query isolates were previously typed employing standard serological techniques. Utilizing pair-wise similarity percentages as calculated by the Pearson correlation coefficient, the predicted serotype of 28 isolates matched the serological typing result. For eight isolates, rep-PCR results were interpreted as one of two very closely-related serotypes, Hadar and the rarer Istanbul. Traditional serological assays have difficulty distinguishing between these groups, and sequencing interspacer regions of the *rrfH* gene was unable to differentiate among isolates of these two serovars. Six of the remaining isolates resulted in no match to the database (similarity values <95%) and these indeed proved to be serotypes not included in the original library. The two remaining samples proved discrepant at the 95% similarity threshold, however examination of electropherograms clearly indicated fingerprint variability between query and library samples, suggesting an expanded rep-PCR library will be necessary for increased utility. Since serological assays can take several days to weeks to provide information, the DiversiLab System holds promise for more rapid serotype classification for members of this group.

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1. Introduction

Members of the genus *Salmonella* have been long recognized as a major cause of gastrointestinal disease in both humans and animals (Darwin and Miller, 1999). Discrimination of *Salmonella* spp. isolates below the species level is imperative for effective epidemiological investigations during outbreak events. The traditional method for subspecies typing among the salmonellae has been serotyping. The serotype is based on the antigenic properties of the O-antigen (surface polysaccharide) and H antigen(s) (flagellar). Typing the O-antigen itself denotes the serogroup, and the serotype is obtained by characterizing the flagella, which is often biphasic (Popoff and Le Minor, 2001). As of 2002, there were approximately 2500 serotypes known, with approximately 1500 serotypes identified within the *Salmonella enterica* subsp. *enterica* (I), the group of salmonellae with clinical relevance whose usual habitat is warm-blooded animals (CDC, 2007). This large number of serotypes is not evenly distributed in nature, however. For example, the top 30 most frequently reported serotypes from human sources account for 81.5% of all isolates reported to the CDC in 2005 (CDC, 2007).

Salmonella are a very well-studied group of organisms and isolates have been examined by a wide array of techniques including ribotyping, phage typing, plasmid profiling, RAPDs and pulsed field gel electrophoresis (Foley et al., 2007; Helmuth and Schroeter, 1994; Lukinmaa et al., 2004). Some of these methods have reported typing resolution at the serotype level, but others such as PFGE offer discrimination within serovars (Fernandez et al., 2003; Foley et al., 2006). Although serotyping has historically proven undeniably useful for investigations, it requires specialized skills and reagents. The categorization of isolates below the serogroup level is performed primarily at a small number of reference laboratories, often with a turnaround time of days or weeks. The ability to rapidly determine serotype with a universally available method would alleviate these delays in response to an outbreak situation (Lukinmaa et al., 2004; Withee and Dearfield, 2007). Consequently, there still remains a need for a reliable method to serologically type salmonellae for source tracking. This particularly important for poultry products which are

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often implicated in *Salmonella*-associated enteric disease in humans. Efforts to reduce contamination of poultry by salmonellae have primarily targeted the processing plant, and while most plants have been able to meet the U.S. Department of Agriculture's Food Safety and Inspection Service (FSIS) mandated hazard analysis and critical control point (HACCP) salmonellae performance standards, several have failed one or two phases of the three phase sampling plan (Altekruse et al., 2006; White et al., 2007).

The repetitive sequence-based PCR method (rep-PCR) uses primers that target non-coding repetitive sequences interspersed throughout the bacterial and fungal genome (Koeuth et al., 1995; Stern et al., 1984; Versalovic et al., 1991). The amplified DNA fragments, when separated by electrophoresis, constitute a genomic fingerprint that can be employed for subspecies discrimination and strain delineation of bacteria and fungi (Versalovic et al., 1993; Versalovic and Lupski, 2002). The development of a commercially available, semi-automated rep-PCR assay system, the DiversiLab System, offers advances in standardization and reproducibility over manual, gel-based rep-PCR (reviewed in [Healy et al., 2005]). Recent studies have described rapid, strain-level discrimination using the DiversiLab System for bacterial genera including *Streptococcus* (Harrington et al., 2007), *Staphylococcus* (Schutt et al., 2005), *Enterococcus* (Sherer et al., 2005), *Bacillus* (Fajardo-Cavazos and Nicholson, 2006), *Escherichia* (Vogel et al., 2007), *Pseudomonas* (Tam, 2007), and *Acinetobacter* (Saeed, 2006). The System allows for archiving of fingerprint patterns using web-based software, and databases created with characterized strains can be used as reference libraries against which unknown samples can be queried. In this report, we created a library of rep-PCR patterns from 13 common and 1 rare *Salmonella* serotypes and subsequently compared 44 blinded unknown *Salmonella* isolates in an attempt to determine the serotype.

2. Materials and methods

2.1. Salmonella strains, culture and DNA extraction

To construct the initial *S. enterica* subspecies *enterica* library, 216 isolates from 14 serotypes in poultry were characterized by the USDA Agricultural Research Service, Bacteriological Epidemiology and Antimicrobial Resistance Research Unit in Athens, Georgia. The serotypes included are among those most commonly isolated from commercial broiler chickens as reported by the USDA-FSIS: [http://www.fsis.usda.gov/Science/Serotypes_Profile_Salmonella_Isolates/index.asp]. In regard to human illness, these groups accounted for over 52% of the serotypes most frequently reported to the CDC isolated from human clinical samples (CDC, 2007). *Salmonella* Kentucky is not pathogenic for humans but is the third most frequently reported serovar from non-clinical nonhuman source in 2005 (CDC, 2007). The reference and query sample isolates were from the National Antimicrobial Resistance Monitoring System (NARMS) Enteric Bacteria collection (Zhao et al., 2005; Foley et al., 2006). Isolates were serotyped by the National Veterinary Services Laboratory (Ames, IA) or with commercial Difco antisera (Becton Dickinson and Company, Sparks, MD) according to the manufacturer's instructions. For DNA extraction, *Salmonella* isolates were cultured on trypticase soy agar (TSA) II with 5% sheep blood for 24 h at 37 °C. DNA from each isolate was extracted using the UltraClean™ Microbial DNA Isolation Kit (Mo Bio Laboratories, Solana Beach, CA) following manufacturer's instructions. Genomic DNA samples were adjusted to approximately 25 ng/µl utilizing UV spectroscopy at 260 nm.

2.2. rep-PCR DNA fingerprinting

All DNA samples were amplified using the DiversiLab *Salmonella* Kit for DNA fingerprinting (bioMerieux, Inc., Durham, NC) following the manufacturer's instructions. Briefly, 2 µl of genomic DNA (concentration approximately 25 ng/µl), 0.5 µl (or 2.5 U) of AmpliTaq® polymerase (Applied Biosystems, Foster City, CA.), 2 µl kit-supplied primer mix and 2.5 µl of 10× GeneAMP PCR Buffer I (Applied Biosystems) were added to 18 µl of the kit-supplied rep-PCR master mix (MM1) for a total of 25 µl/PCR reaction mixture. Thermal cycling parameters were as follows: initial denaturation of 94 °C for 2 min; followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, extension at 70 °C for 90 s; and a final extension at 70 °C for 3 min. Detection of rep-PCR products was implemented using the DiversiLab System (bioMerieux, Inc.), which employs a microfluidics chip-based DNA fragment separation rather than gel electrophoresis traditionally employed for rep-PCR analyses. Thirteen samples can be analyzed simultaneously on a microfluidics chip and internal DNA standards of known sizes are added to each well to allow for normalization and efficient chip-to-chip comparisons.

2.3. Analysis and library construction

Analysis was performed with the DiversiLab software version 3.3 and the resulting DNA fingerprint patterns were viewed as electropherograms, and the reports included a dendrogram constructed from a similarity matrix and a virtual gel image of the fingerprint for each DNA sample. All library entries were typed in duplicate and the observed reproducibility was consistent. For purposes of predicting serotype on the basis of rep-PCR fingerprints, the Top Match feature of the DiversiLab software was employed. Top Match can be used with DiversiLab classification reports to illustrate the five most similar entries in a library to a query sample. If the query sample matched a particular serotype library entry at >95% similarity, it was considered to be a positive designation. Since the isolates of closely-related serotypes Istanbul and Hadar could not be totally distinguished by rep-PCR, nor reliably differentiated by classical serotyping or sequencing techniques (see below), isolates originally identified as members of these serotypes were considered one group.

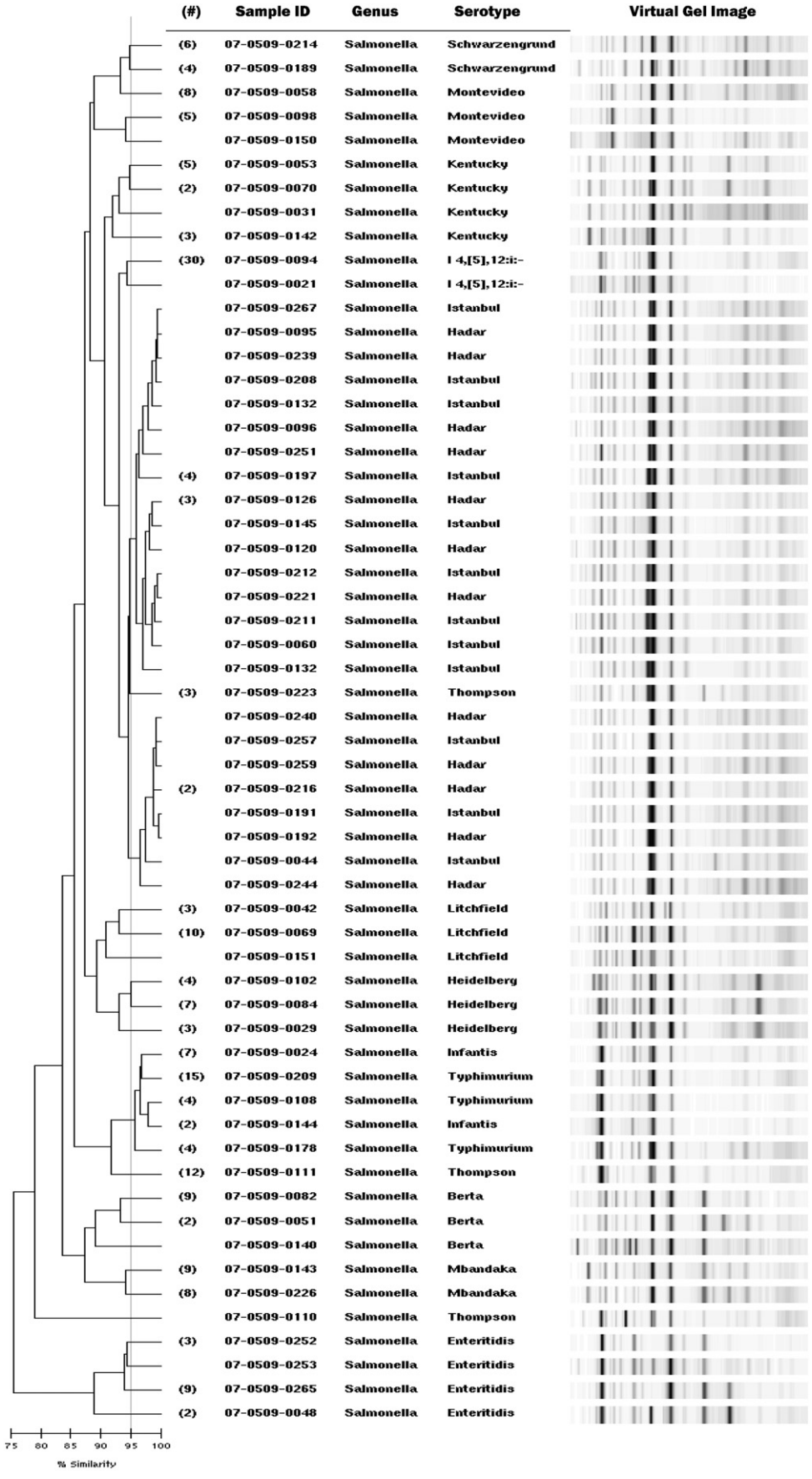
2.4. DNA sequencing

Primers ISRH-1 and ISRH-2 were utilized to amplify the two intervening spacer regions (ISRs) between the 23 S *rrlH* gene and *yafB* gene as described (Morales et al., 2006). The amplicons were purified using the Qiagen PCR purification kit (Qiagen, Maryland). Bidirectional sequence data were generated using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems). The products were purified using ethanol precipitation, and sequenced using an ABI3730 sequencer (Applied Biosystems). Contiguous sequences were assembled and edited using the SeqMan software program, which is part of the Lasergene suite of sequence analysis software (DNASTAR, Inc., Madison, Wis.). Sequences were aligned using the ClustalW function of the MegAlign program (DNASTAR, Inc.).

3. Results

3.1. Library construction of S. enterica subsp. enterica isolates from poultry

All *Salmonella* isolates were capable of being typed with the DiversiLab System and *Salmonella* DNA fingerprinting kit. For analysis, DiversiLab software utilized the Pearson correlation coefficient and the unweighted pair-group methods of averages (UPGMA) to automatically compare the rep-PCR profiles and create corresponding dendrograms (Healy et al., 2005). In addition, reports included computer-generated virtual gel images and selected demographic fields to aid interpretation of the data. The dendrogram generated from rep-PCR patterns generally illustrated clustering by serotype (Fig. 1). There were only two cases where isolates of different serotypes showed fingerprints that were >95% similar, Hadar/Istanbul


(#) Sample ID Genus Serotype Virtual Gel Image
(6) 07-0509-0214 Salmonella Schwarzengrund
(4) 07-0509-0189 Salmonella Schwarzengrund
(8) 07-0509-0058 Salmonella Montevideo
(5) 07-0509-0098 Salmonella Montevideo
07-0509-0150 Salmonella Montevideo
(5) 07-0509-0053 Salmonella Kentucky
(2) 07-0509-0070 Salmonella Kentucky
07-0509-0031 Salmonella Kentucky
(3) 07-0509-0142 Salmonella Kentucky
(30) 07-0509-0094 Salmonella I 4,[5],12:i:-
07-0509-0021 Salmonella I 4,[5],12:i:-
07-0509-0267 Salmonella Istanbul
07-0509-0095 Salmonella Hadar
07-0509-0239 Salmonella Hadar
07-0509-0208 Salmonella Istanbul
07-0509-0132 Salmonella Istanbul
07-0509-0096 Salmonella Hadar
07-0509-0251 Salmonella Hadar
(4) 07-0509-0197 Salmonella Istanbul
(3) 07-0509-0126 Salmonella Hadar
07-0509-0145 Salmonella Istanbul
07-0509-0120 Salmonella Hadar
07-0509-0212 Salmonella Istanbul
07-0509-0221 Salmonella Hadar
07-0509-0211 Salmonella Istanbul
07-0509-0060 Salmonella Istanbul
07-0509-0132 Salmonella Istanbul
(3) 07-0509-0223 Salmonella Thompson
07-0509-0240 Salmonella Hadar
07-0509-0257 Salmonella Istanbul
07-0509-0259 Salmonella Hadar
(2) 07-0509-0216 Salmonella Hadar
07-0509-0191 Salmonella Istanbul
07-0509-0192 Salmonella Hadar
07-0509-0044 Salmonella Istanbul
07-0509-0244 Salmonella Hadar
(3) 07-0509-0042 Salmonella Litchfield
(10) 07-0509-0069 Salmonella Litchfield
07-0509-0151 Salmonella Litchfield
(4) 07-0509-0102 Salmonella Heidelberg
(7) 07-0509-0084 Salmonella Heidelberg
(3) 07-0509-0029 Salmonella Heidelberg
(7) 07-0509-0024 Salmonella Infantis
(15) 07-0509-0209 Salmonella Typhimurium
(4) 07-0509-0108 Salmonella Typhimurium
(2) 07-0509-0144 Salmonella Infantis
(4) 07-0509-0178 Salmonella Typhimurium
(12) 07-0509-0111 Salmonella Thompson
(9) 07-0509-0082 Salmonella Berta
(2) 07-0509-0051 Salmonella Berta
07-0509-0140 Salmonella Berta
(9) 07-0509-0143 Salmonella Mbandaka
(8) 07-0509-0226 Salmonella Mbandaka
07-0509-0110 Salmonella Thompson
(3) 07-0509-0252 Salmonella Enteritidis
07-0509-0253 Salmonella Enteritidis
(9) 07-0509-0265 Salmonella Enteritidis
(2) 07-0509-0048 Salmonella Enteritidis
75 80 85 90 95 100
% Similarity

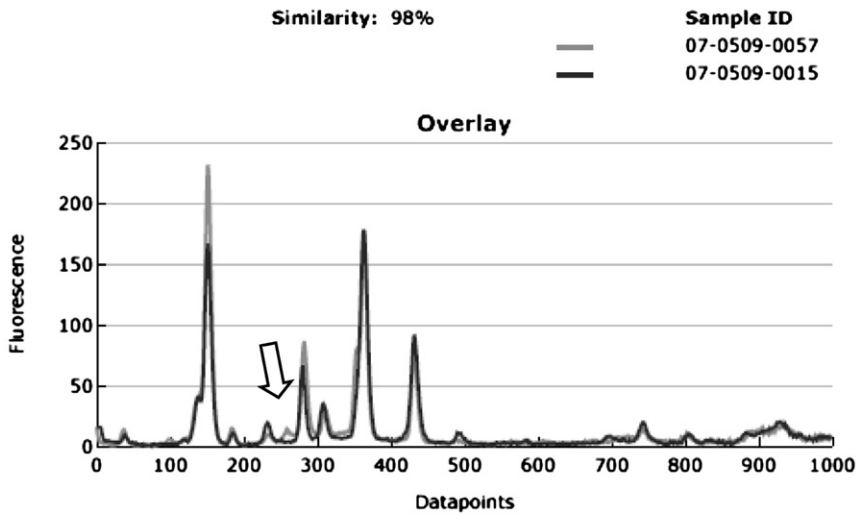


Fig. 2. Electropherogram overlay of rep-PCR amplicons from *Salmonella* Typhimurium isolate 07-0509-0015 (black curve) and *Salmonella* Infantis isolate 07-0509-0057 (grey curve). The arrow indicates a fluorescent peak difference between the samples. This peak was consistently present among all Infantis in the library and absent among Typhimurium.

and Infantis/Typhimurium. However, a close examination of the DiversiLab electropherograms demonstrated that there was a small but consistent peak difference among all the Infantis and Typhimurium isolates (Fig. 2). A number of rep-PCR fingerprints generated from isolates originally serotyped as Istanbul and Hadar were indistinguishable from each other. This result is not surprising, as these serovars are closely related. In fact, the antigenic formula are very similar, Hadar is defined as **I 6,8:z10:e,n,x**, whereas Istanbul only lacks the O-antigen 6, with both phases of the flagellar antigens the same: **I 8:z10:e,n,x**. Several library entries originally classified as Istanbul were sent to a reference lab for confirmation, and the isolates were returned as Hadar. The inconsistent serotyping results may be explained by differential expression of the O-antigen epitope "6". For example, one isolate could be glucosylated to meet a threshold of reactivity or one of them could be better at linking a decoration such as an acetyl group to the glucose moiety (Guard-Petter, 1999).

The inconsistent serotyping was further investigated with DNA sequencing. The interspacer regions between the end of the 23S *rRNA* gene and start of 5S *rRNA* gene (ISR-1), and the end of the 5S *rRNA* gene and the beginning of the transfer rRNA *aspU* (ISR-2) in one of the ribosomal rRNA operons in *Salmonella*, *rrnH*, have been shown to differentiate among closely-related *Salmonella* serotypes, including the avian-adapted Gallinarum and Pullorum (Morales et al., 2006). Ten isolates each of serovar Istanbul and Hadar were examined and shown to have identical sequence in these genomic regions. Thus, taken in combination with the inconsistent serotyping, the Hadar and Istanbul isolates were considered one group when comparing unknowns to the library.

For certain serotypes, there was limited variability within the group. For example, 30 of the 31 I 4,[5],12:i:− fingerprints were >95% similar to each other (Fig. 1). However, most serovars exhibited more extensive strain-level discrimination when placed in the dendrogram. For example, three discrete fingerprint types were evident with *Salmonella* Thompson. The group represented by isolate 07-0509-0111 in Fig. 1 was on average 83% similar to the cluster represented by isolate 07-0509-0223. Serotype Thompson isolate 07-0509-0110 was only on average 77% similar to the 07-0509-0111 group, and 88% similar to the 07-0509-0223 cluster. This result suggests that the DiversiLab *Salmonella* DNA fingerprinting kit may be useful for outbreak investigation of certain serovars when a sub-serotype level of discrimination is necessary for source tracking. The cell surface antigenic formulation of any one *Salmonella* serotype is an end outcome determined by its genomic content expression in response to its environment. Serovars that had multiple rep-PCR patterns, like Thompson, may be accumulating substantial genomic change more rapidly than other serotypes; however, none of the genomic rearrangements detected by rep-PCR altered the expression of genes that determine its antigenic formula. Alternatively, the three separate *S. enterica* lineages may have evolved similar antigenic formulations as a function of evolutionary convergence due to common selective pressure.

3.2. Identification of Salmonella serotypes by comparison to the library

Forty-four *Salmonella* isolates obtained from the USDA as part of the NARMS collection were typed with the DiversiLab System and queried against the *Salmonella* library described herein. The blinded isolates were serotyped by standard assays (Popoff and Le Minor, 2001). The clustering of the DiversiLab dendrogram and the Top Match function of DiversiLab classification report was subsequently utilized as a guide to predict serotype. Using 95% similarity as a threshold, the library matches and putative serotypes are listed in Table 1. Of the 38 isolates from which a putative serotype match was obtained using the library, 36 were in concordance with the traditional serotyping result (94.7%). For the six samples that returned no match to the database (similarly values <95%), all six proved to be serotypes not included in the original library. An example of the top match output created by the software is illustrated in Fig. 3.

The remaining two discrepant samples emphasize the importance of a close examination of the electropherograms generated by the software. Sample analysis of unknown #25 showed it to be 96.3% similar to 07-0509-0190 in the database, a Schwarzengrund serotype. Traditional serotyping revealed that the isolate was Bredeney, also a member of O-serogroup "B". Query sample #07 was 96.8% similar to 07-0509-0064 (Kentucky) and conventional serotyping returned a result of Lille. The electropherograms for each of these two query samples and the database Top Match are provided in Fig. 4. It is

Fig. 1. Dendrogram illustrating the DiversiLab library of 216 poultry-associated *Salmonella* spp. isolates. The dendrogram was collapsed due to space constraints such that only one fingerprint profile of groups of library entries of the same serotype at >95% similarity is shown. The number of samples at collapsed nodes is indicated in parenthesis. The Pearson's correlation coefficient was used to generate a pair-wise percent similarity matrix, and the tree was created using the unweighted pair group method of arithmetic averages. The horizontal scale bar indicates the percent similarity among strains.

Table 1
List of *Salmonella* isolates with predicted serotype and serological results

Query sample #	Predicted serotype by rep-PCR	Similarity % of Top Match	Traditional serology result
01	Hadar/Istanbul	99.1	Istanbul
02	4,[5],12:i–	97.3	4,5,12:i:–
03	Enteritidis	98.5	Enteritidis
04	No match	91.2	Derby
05	Kentucky	95.8	Kentucky
06	Enteritidis	97	Enteritidis
07	Kentucky	96.8	Lille
08	Hadar/Istanbul	97.5	Hadar
09	Schwarzengrund	98.4	Schwarzengrund
10	Typhimurium	98.4	Typhimurium
11	Hadar/Istanbul	97.1	Hadar
12	Montevideo	98	Montevideo
13	Kentucky	96.8	Kentucky
14	4,[5],12:i–	98.7	4,5,12:i:–
15	Thompson	97.9	Thompson
16	No match	92.1	Kiambu
17	Berta	98	Berta
18	Mbandaka	98.5	Mbandaka
19	Infantis	98.2	Infantis
20	Montevideo	99	Montevideo
21	Heidelberg	97.5	Heidelberg
22	Typhimurium	98	Typhimurium
23	No match	87.4	Johannesburg
24	Mbandaka	96.8	Mbandaka
25	Schwarzengrund	96.3	Bredeney
26	Schwarzengrund	95.1	Schwarzengrund
27	Berta	97.4	Berta
28	Thompson	99	Thompson
29	Heidelberg	99	Heidelberg
30	Infantis	98.4	Infantis
31	Litchfield	98.2	Litchfield
32	Hadar/Istanbul	98.1	Hadar
33	No match	94.9	Cerro
34	Infantis	97.7	Infantis
35	4,[5],12:i–	97.7	4,[5],12:i–
36	Berta	97.3	Berta
37	Heidelberg	98.3	Heidelberg
38	No match	79.8	Worthington
39	No match	86.6	Rissen
40	Enteritidis	97.9	Enteritidis
41	Hadar/Istanbul	98.3	Istanbul
42	Hadar/Istanbul	99	Istanbul
43	Litchfield	98.8	Litchfield
44	Hadar/Istanbul	98.7	Hadar

apparent that there were many differences among the amplicons generated with the DiversiLab *Salmonella* kit. Therefore caution is advised when employing the 95% threshold value when comparing to a preexisting library.

4. Discussion

Herein we report that the semi-automated rep-PCR assay was capable of reliably predicting *Salmonella* spp. serotype from a set of unknown isolates relative to a continuing development of well-characterized standards. Manual rep-PCR has previously been applied to *Salmonella* isolates for the purpose of identifying serotypes with mixed results reported. Van Lith and Aarts (1994) showed that the ERIC (enterobacterial repetitive intergenic consensus sequence) primer set produced serotype-specific fingerprints. Employing a 3-primer composite rep-PCR, Weigel et al. (2004) observed that tight clusters were 82% homogeneous for serotype. Some serotypes, however, were dispersed in multiple clusters. Johnson et al. (2001) observed resolution at the serotype level with representatives of 15 serotypes and suggested that rep-PCR with ERIC2 and BOXA1R primers could be used as a rapid and simple replacement for serotyping. However, Millemann (1996) reported that ERIC-PCR could not discriminate between some Enteritidis and Typhimurium isolates, and Burr et al. (1998), stated that in their hands ERIC-PCR was not useful for identifying serotypes. These authors commented that matching fingerprints from different gels was difficult, and it is uncertain to what extent this manual methodology affected the results. Rasschaert et al. (2005) concluded that ERIC and the (GTG)5 primers both provided serotype discrimination and rep-PCR offered utility for reducing serotyping load during an outbreak. However, these investigators also reported that there were reproducibility issues that again may be attributed to the manual methodology. The DiversiLab System obviates the need to compare separate gels, as the amplicons are separated on microfluidics chips with internal standards added to each sample well (Healy et al., 2005).

The semi-automated rep-PCR based method evaluated for determining serotypes of *Salmonella* correctly classified 36 of 44 unknowns. Consequently, an extended *Salmonella* database of rep-PCR patterns will be required to eventually replace conventional serotyping procedures. Therefore, we currently envision this method as a first screening to limit the number of antisera that need to be tested against a particular unknown. The traditional antigen–antibody-based Kauffmann–White serotyping scheme requires the use of approximately 250 antisera (Popoff et al., 2003), and a rapid screen could greatly reduce the technician time and reagent volume necessary to definitively determine *Salmonella* serotypes. After acquisition of capital equipment, the cost of the rep-PCR typing is comparable to the standard fee charged by reference laboratories. Furthermore, molecular methods, like rep-PCR allow for typing results of mutant strains, including “rough” variants that do not express O-antigen, or non-motile isolates that express no H antigens. In conclusion, we have demonstrated that rep-PCR can be utilized to reliably and rapidly predict *Salmonella* serotype as a substitute for the standard serological assay. The goal is to continue development of an expanded, more comprehensive rep-PCR fingerprint library that will help facilitate more accurate serotype designations for unknown *Salmonella* isolates to support epidemiological analyses.

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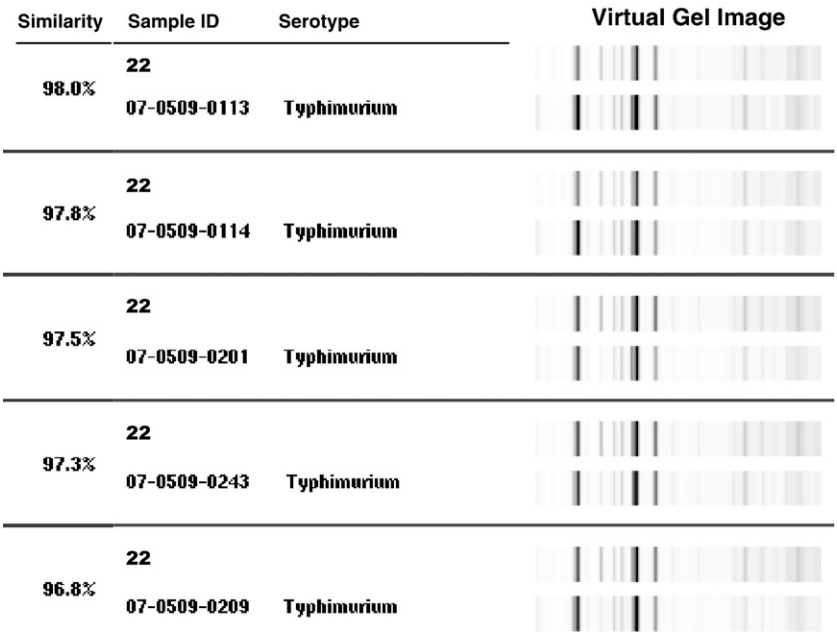

Fig. 3. Top match report generated by the DiversiLab software. Uncharacterized *Salmonella* isolate #22 was queried against the poultry-associated *Salmonella* library and the five most similar library entries along with serotype designation and virtual gel images are displayed.

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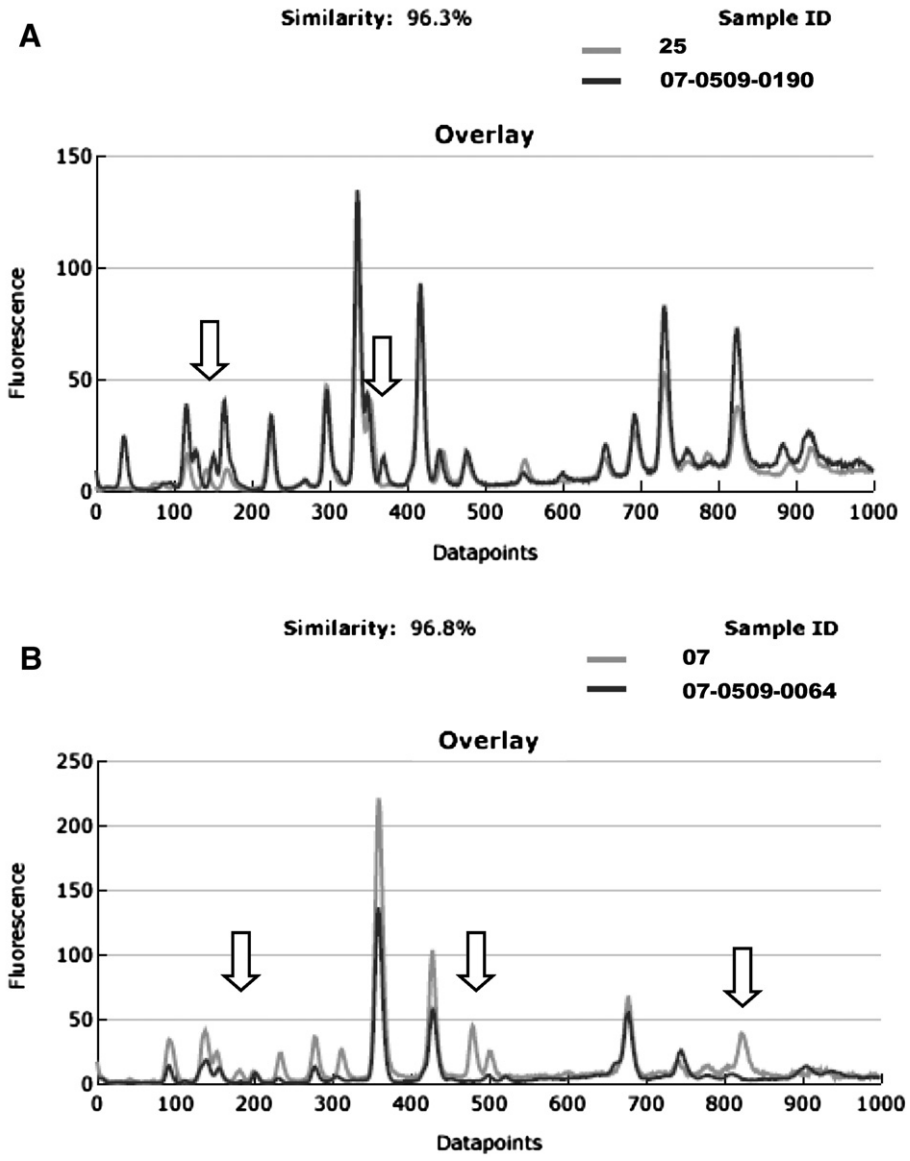

Fig. 4. Electropherogram overlays of uncharacterized query samples #25 (panel A) and #07 (panel B) and their respective top matches in the poultry *Salmonella* library. The arrows indicate regions of peak differences between the samples. For (A), unknown #25 was serotyped as "Bredeney" and the library top match 07-0509-0190 was "Schwarzengrund". For (B) unknown #07 was serotyped as "Lille" and the library top match 07-0509-0064 was "Kentucky".